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Breast Cancer Risk

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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research is to develop a specific monoclonal antibody (MAb) for intracellular detection and quantification of E_2-3,4-Q adducted to proteins, as a potential biomarker of breast cancer risk. An established, protein-coupling chemistry was applied successfully to production of an immunogen, *via* linkage of E_2-3,4-Q to cationized protein, through a short linker. A coating complex for indirect ELISA and a low molecular-weight conjugate for competitive ELISA were prepared by spontaneous adduction of E_2-3,4-Q to an unmodified protein and a short-chain molecule with a primary amino group, respectively. ELISA screening identified a hybridoma producing relatively large amounts of MAb with high signal-to-noise ratio. The affinity constant (0.5×10^8 M^{-1}) for binding to E_2-3,4-Q was determined by competitive ELISA. In specifically designed immunohistochemical (IHC)-staining protocols, the antibody produced intense and specific staining with no detectable cross-reactivity to E_2-2,3-Q and little or no background. Failure to detect IHC staining in paraffin sections of ACI rat breast tumors or human breast tumors is associated with loss of the epitope during microwave recovery or formalin fixation. Optimization of tissue fixation is required for validation studies that can be based on Quantitative Fluorescence Imaging Analysis or Surfaced-Enhanced Laser Desorption Ionization/tandem mass spectrometry.

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INTRODUCTION

Breast cancer, the leading cause of cancer-related deaths among non-smoking women, is linked to cumulative estrogen exposure. A compelling body of information supports a close association between estrogen-dependent carcinogenesis (E-DC) and preferential 4-hydroxylation of 17beta-estradiol (E_2) to form 4-OHE$_2$, in both model systems and human breast tissue (1). Formation of E_2-3,4-quinone (E_2-3,4-Q) the oxidation product of 4-OHE$_2$ is thought to be essential for E-DC of the breast and can contribute to E-DC *via* indirect or direct damage to DNA. In tissues susceptible to E-DC, 4-OHE$_2$ and E_2-3,4-Q may escape detoxification, *e.g.*, methylation of the catechol or glutathione conjugation of the quinone, and form E_2-3,4-Q adducts to proteins. The presence of these adducts in nipple aspirate or fine-needle aspirate cells may serve as a biological marker of non-detoxified E_2-3,4-Q and breast cancer risk. These considerations have prompted the present proposal aimed at a) producing a monoclonal antibody (MAb) specific for E_2-3,4-Q adducted to any protein, and b) evaluating its immunochemical and immunohistochemical characteristics.

BODY

A. Synthesis of an immunogen consisting of estradiol-3,4-quinone (E_2-3,4-Q) covalently linked to hen egg albumin (OA)

1. Basis for the idea that E_2-3,4-Q could form simple and stable adducts to protein amino groups

The literature contains no published studies of either spontaneous adduction or chemical coupling of E_2-3,4-Q to proteins. The possibility of spontaneous formation of simple and stable adducts of E_2-3,4-Q to amino side groups (primarily ε-amino groups of lysine residues) of proteins was conceived in light of 1) the electrophilic nature of carbon-1 of E_2-3,4-Q and its reactivity with mimics of amino acid side chains (2), and 2) published studies of spontaneous adduction of urushiol quinones to human serum albumin and 1-aminopentane a model primary amine (3). Liberato *et al.* (3) demonstrated regiospecific attack of 1-amino-pentane at carbon-5 of 3-heptadecylbenzoquinone, producing the aminoquinone, 3-heptadecyl-5-(pentylamino)-1,2-benzoquinone (**Figure 1**). The reaction produced a characteristic red shift in the absorption spectrum of the quinone. The aminoquinone had an absorption maximum of 480 nm and exhibited a distinctive red color. The authors concluded, that urushiol quinones (with configurations analogous to the estrogen quinones) react efficiently with primary amino groups of HSA
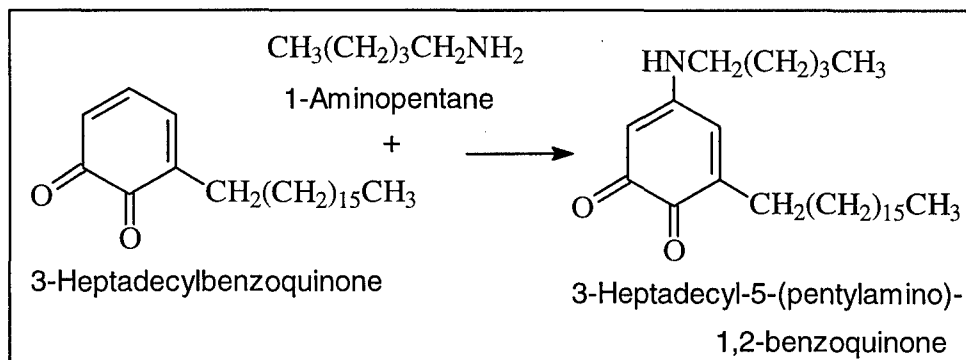

Figure 1

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thus producing aminoquinones represented by 3-heptadecyl-5-(pentylamino)-1,2-benzoquinone.

The work of Liberato *et al.* (3) prompted a study of the reaction of E_2-3,4-Q and E_2-2,3-Q with N-acetyl-L-lysine (N-AcLys) in acetic acid/water (4). E_2-3,4-Q a yellow chromophore reacted efficiently with N-AcLys, producing estradiol-3,4-quinone-1-N-acetyl-L-lysine (E_2-3,4-Q-1-N-AcLys) with a 73% yield (**Figure 2**). Genesis of E_2-3,4-Q-1-N-AcLys in the reaction mixture produced a characteristic red shift in the absorption spectrum of the quinone. The aminoquinone exhibited a distinctive red color that permits convenient monitoring of the occurrence and progression of the reaction and quantification of the aminoquinone.
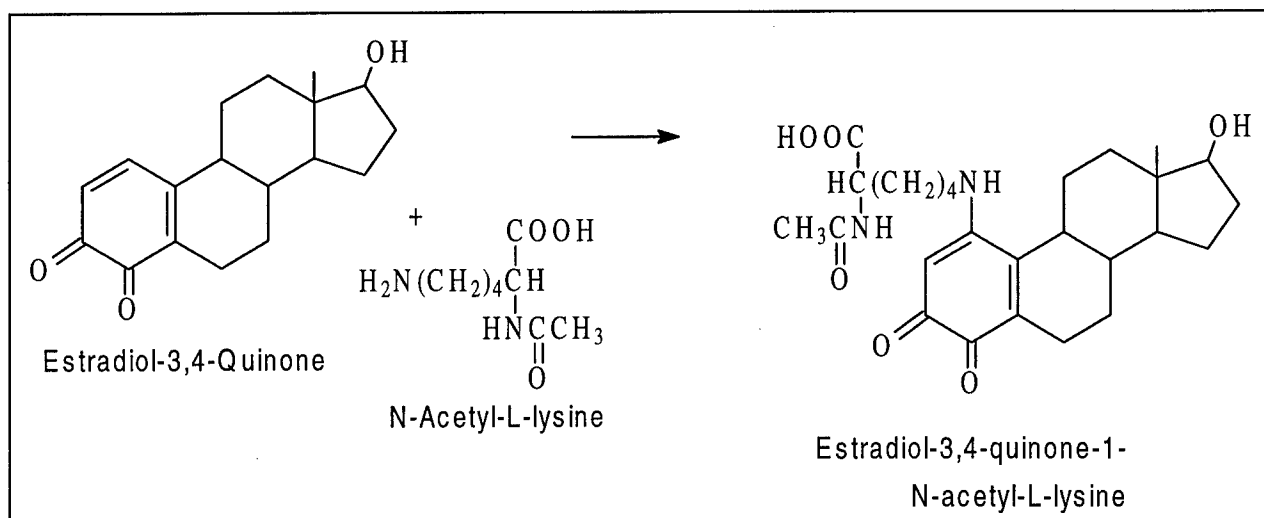

Figure 2

Reaction of E_2-2,3-Q with N-AcLys produced 2-hydroxyestradiol-6-N-acetyl-L-lysine (2-OHE$_2$-6-N-AcLys) with a 38% yield (**Figure 3**). The latter is not a chromophore, consequently the yellow color (due to E_2-2,3-Q) of the reaction mixture faded as the reaction proceeded.
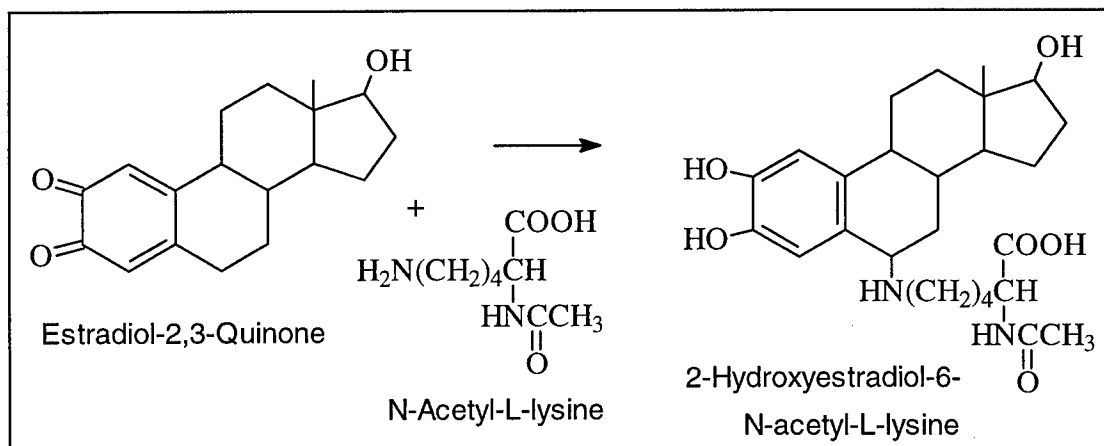

Figure 3

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This work demonstrated the rather clean reaction of E_2-3,4-Q and E_2-2,3-Q with primary amino groups in acetic acid/water and supported the possibility of direct adduction of the estrogen quinones with proteins and development of a monoclonal antibody that could easily discriminate between amino-adducted E_2-3,4-Q and E_2-2,3-Q. Significant questions,however, remained unanswered. Does E_2-3,4-Q form adducts to primary amino groups of model compounds and to proteins, at or near physiological pH? Does the reaction of E_2-3,4-Q with primary amino groups occur efficiently, only in highly acidic environments? Does direct adduction of E_2-3,4-Q to proteins occur at a number of sites sufficient to produce an effective immunogen needed for induction of anit-E_2-3,4-Q specific antibodies, and a coating complex needed for immunochemical studies of antibody potency and specificity? Do steric limitations posed by fixed positioning of reactive nucleophiles (*e.g.*, primary amino groups) in relation to other amino acid residues on proteins disfavor useful or spontaneous adduction of E_2-3,4-Q?

2. Principles guiding production of an immunogen that would induce antibodies specifically reactive with stable adducts of E_2-3,4-Q to proteins or other macromolecules

a. A low-molecular-weight protein [hen egg albumin (OA)], in comparison to a high molecular-weight protein, *e.g.*, keyhole limpet hemocyanin (KLH), is a superior carrier for immunization against E_2-3,4-Q. The investigator has determined in previous studies that sufficient substitution of KLH with a hydrophobic hapten, such as E_2-3,4-Q, produces an unmanageable paste. Limiting the rate of haptenation of KLH will produce an immunogen that may elicit anti-E_2-3,4-Q clones as a small minority against the background of a wide diversity of clones against relatively potent KLH-specific epitopes. In contrast, high-frequency haptenation of OA with a hydrophobic hapten will produce a manageable precipitate that can be washed and resuspended in buffer. The fine precipitate acts as an immuno-adjuvant and at the same time, relevant epitope (E_2-3,4-Q) frequency is high.

b. The immunogen must be produced by either direct adduction of E_2-3,4-Q to protein or coupling *via* a <u>short</u> linker. Preliminary studies demonstrated spontaneous formation of E_2-3,4-Q adducts to both OA and bovine serum albumin (BSA) in phosphate buffer at pH 6.5. Thus, supporting the feasibility of spontaneous adduction of endogenously generated E_2-3,4-Q to cellular proteins. These adducts will be in close proximity to the protein surface and are expected to be recognized by antibodies whose binding "pockets" are relatively shallow. Immunization of mice with immunogen formed by either direct adduction of E_2-3,4-Q or coupling *via* a short linker will favor induction of efficient antibodies with "shallow" binding sites. The use of long-arm linkers would allow induction of antibodies with deep, binding "pockets" that may be sterically hindered in their approach to adducts formed spontaneously on cellular proteins.

c. The immunogen will induce antibodies that recognize E_2-3,4-Q but not E_2-2,3-Q. Carcinogenesis studies support E_2-3,4-Q as the principal carcinogen inducing the majority of breast cancers. Both E_2-3,4-Q and E_2-2,3-Q can be formed in breast epithelial cells. The corresponding catechols (4-OHE$_2$ and 2-OHE$_2$) have been detected in normal and cancerous breast tissue from women, however, 4-OHE$_2$ the precursor to E_2-3,4-Q is more abundant in cancerous breast tissue. The reactions of E_2-3,4-Q and E_2-2,3-Q with primary amino groups (abundant nucleophiles of proteins) assure that antibodies induced against E_2-3,4-Q adducted to protein will react poorly if at all with E_2-

2,3-Q adducted to protein (**Figure 4**). Linkage at carbon-1 of E_2-3,4-Q (represented by E_2-3,4-Q-1-N-GABA) and at carbon-6 of E_2-2,3-Q (represented by 4-OHE$_2$-6-N-GABA) produce markedly different molecular configurations these adducted quinones present to receptors of the immune system (**Figure 4**). This is in contrast to potential cross-reactivity of induced antibodies with <u>free</u> E_2-2,3-Q, which is of no consequence for immunohistochemical detection of protein-adducted E_2-3,4-Q. Free catechols and quinones are expected to be present at very low concentrations (a few pmols per mg tissue) and will be removed during processing of tissues and cells for immunohistochemistry.
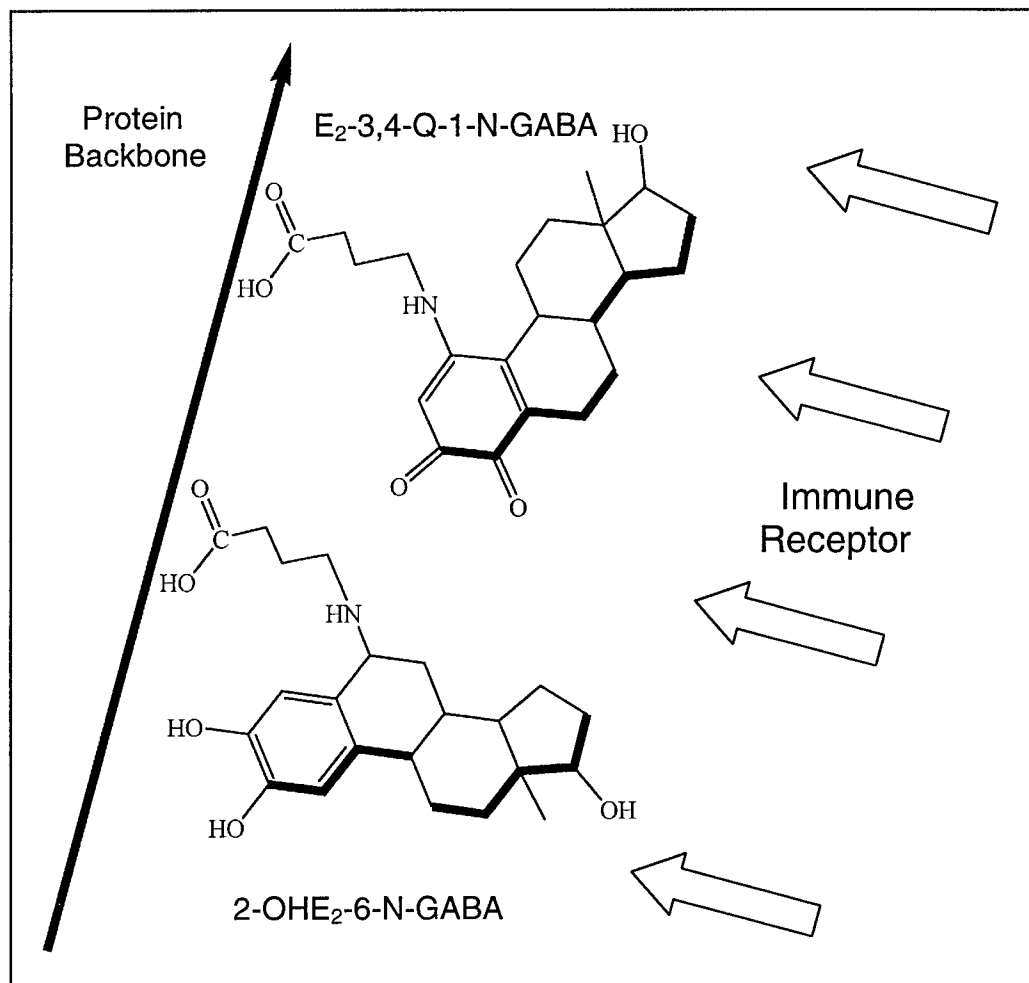

Figure 4

3. Synthesis I: Direct coupling of E_2-3,4-Q to hen egg albumin (OA)
a. Preparation of E_2-3,4-Q

E_2-3,4-Q was prepared from 4-hydroxyestradiol (4-OHE$_2$) by standard procedures, immediately prior to use. Three milligrams (mg) of 4-OHE$_2$ were dissolved in 3.0 milliliters (mL) of aceonitrile (CH$_3$CN) by sonication. This produced a clear and colorless solution. Sodium acetate buffer (pH 6.5) and manganese oxide (MnO$_2$) were added to the solution and the mixture was vortexed every minute (min) for 10 min. The

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mixture was filtered with a 0.45 µ PTFE filter and the clear yellow filtrate (E$_2$-3,4-Q disolved in CH$_3$CN) was transferred to a 25 mL rotary evaporator flask. Dimethylformamide (DMF, 1.5 mL) was added to the flask and mixed with the E$_2$-3,4-Q solution. CH$_3$CN was evaporated off by rotary evaporation leaving E$_2$-3,4-Q dissolved in DMF/acetate buffer.

b. Coupling E$_2$-3,4-Q to OA

E$_2$-3,4-Q was directly linked to OA, under conditions developed as part of the present research initiative. Procedures for direct coupling of E$_2$-3,4-Q are not published in the scientific literature. A solution of 72 mg OA dissolved in 20 mL phosphate buffer at pH 6.5 (PB6.5) was dialyzed against PB6.5. Three-hundred microliters (µL) of E$_2$-3,4-Q solution was added to each 2.5 mL of dialyzed OA and the reaction mixture was stirred for 15-20 hours (hr) at room temperature. The clear reaction mixture changed from a yellow color to a characteristic red color that indicates coupling of the quinone to the protein. Non-reacted E$_2$-3,4-Q was extracted into cold acetone and the precipitated protein was dried and stored as a dry powder under vacuum. A solution produced by dissolving a small quantity of dried product in phosphate buffered saline, exhibited a characteristic red color indicative of adducted E$_2$-3,4-Q.

c. Estimation of the degree of substitution

The reaction between E$_2$-3,4-Q and a large, molar excess of N-acetyl-L-lysine (N-AcLys) served as a reference reaction for the reaction between E$_2$-3,4-Q and OA. Change in the color of both reaction mixtures was the same and the reaction products had the same absorption maximum at 510 nm. Assuming quantitative coupling of E$_2$-3,4-Q to N-AcLys, the aminoquinone had an extinction coefficient (in buffer, at 510 nm) of 6.55×10^{-4} M. The molar ratio of E$_2$-3,4-Q/OA calculated with this coefficient was 2:1.

4. Synthesis II: Coupling E$_2$-3,4-Q to OA *via* the linker gamma-aminobutyric acid (GABA).

In this procedure, E$_2$-3,4-Q was coupled to gamma-aminobutyric acid (GABA) and then the E$_2$-3,4-Q-GABA complex was linked to amino side groups of cationized OA (catOA) in a reaction catalyzed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy-sulfosuccinimide (S-NHS). The conditions of this procedure were developed as part of the present program and this coupling of E$_2$-3,4-Q to protein is unique. Procedures for coupling E$_2$-3,4-Q to protein *via* a linker are not published in the scientific literature.

Cationization of OA markedly increases the antigenicity of the protein and provides many more amino groups for EDC-catalyzed coupling of the E$_2$-3,4-Q-GABA complex. A solution of 50 mg OA was dissolved in 5 mL PB6.5 and dialyzed against PB6.5. The cationization reaction mixture included 2.0 mL OA solution, 200 mg of ethylenediamine HCl (EDHCl), 114 mg EDC, and 3.3 mg S-NHS. The mixture was stirred with a magnetic bar for 2 hr, at room temperature, and then dialyzed against PB6.5. A small volume of the catOA solution treated with an excess of EDC and S-NHS did not produce a precipitate, indicating efficient cationization of OA.

Production of the E$_2$-3,4-Q-GABA complex is unique to the present program. The reaction mixture included 57 mg GABA dissolved in 15 mL PB6.5 and 1.8 mL E$_2$-3,4-Q solution. The mixture was stirred for 2 hr, at room temperature and then extracted with chloroform. Unreacted E$_2$-3,4-Q was extracted into the organic phase as evidenced by its

yellow color. The red, aqueous layer containing the E_2-3,4-Q-GABA complex, was evaporated to dryness. The dried powder was dissolved in 50% methanol (MeOH)/50% H_2O, and unreacted GABA was removed with a one-gram reverse-phase cartridge (SepPak tC18). The red, cartridge effluent in 100% MeOH was evaporated to dryness and the purified E_2-3,4-Q-GABA complex was stored as a dry powder.

Coupling E_2-3,4-Q-GABA to catOA is unique to the present program. The coupling reaction mixture contained 3.0 mL catOA solution, all of the purified E_2-3,4-Q-GABA prepared as described in the previous paragraph, 114 mg EDC, and 3.3 mg S-NHS. The mixture was stirred with a magnetic bar for 2 hr, at room temperature, and then dialyzed against PB6.5. The dialysis bag contained a red suspension that was separated by centrifugation into a deep red precipitate and a red supernatant. The latter was combined with cold acetone, lyophilized to a dry powder that exhibited a red color (E_2-3,4-Q-GABA-catOA), and stored under vacuum. Spectral analysis of the soluble conjugate at 510 nm indicated that the molar ratio of E_2-3,4-Q to catOA was 10:1. The red precipitate (more highly substituted E_2-3,4-Q-GABA-catOA) was dried and then stored under vacuum.

B. Synthesis of a coating complex consisting of E_2-3,4-Q covalently linked to bovine serum albumin (BSA)
1. Rationale

The coating complex, a unique product of the present research initiative, was produced by direct reaction of E_2-3,4-Q with BSA and not by coupling through the GABA linker. The complex serves in the enzyme-linked immunosorbent assay (ELISA) as an immobilized carrier of E_2-3,4-Q epitopes that will be recognized by specific antibodies. These stable epitopes closely mimic stable E_2-3,4-Q adducts that may form with intracellular or other proteins, thus selecting for antibodies that react well with endogenously formed adducts. In addition, since catOA and BSA exhibit little immunologic cross-reactivity, the use of catOA as immunogen and BSA as coating complex will enhance selection for antibodies of high specificity for E_2-3,4-Q and selection against antibodies recognizing epitopes consisting of E_2-3,4-Q and neighboring elements of the catOA surface. Thus, antibodies that bind with high affinity to E_2-3,4-Q-BSA and do not bind to non-adducted BSA will effectively recognize stable adducts of E_2-3,4-Q to any protein or other macromolecule.
2. Synthesis of the coating complex, E_2-3,4-Q-BSA

The coating complex was produced by direct reaction of E_2-3,4-Q with BSA. The reaction mixture consisted of 11 mg of BSA dissolved in 2.5 mL of PB6.5 and dialyzed against PB6.5 and 300 µg of E_2-3,4-Q dissolved in 300 µL of DMF. The reaction mixture was stirred at room temperature, for 15-20 hr. The conjugated protein was precipitated in cold acetone, washed 3 times with cold acetone to remove unreacted E_2-3,4-Q, and then lyophilized. Spectral analysis of the conjugated protein indicated that the molar ratio of E_2-3,4-Q to BSA was 5:1. The pink-colored, E_2-3,4-Q-adducted protein was stored as a dry powder, under vacuum.

C. Synthesis of a low-molecular-weight competitor consisting of gamma-aminobutyric acid (GABA) linked *via* its primary amino group to carbon-1 of E_2-3,4-Q
1. Discussion

A published study (4) of the reaction of E_2-3,4-Q with N-AcLys, a model compound containing a primary amino group, demonstrated a clean reaction producing E_2-3,4-Q-1-N-AcLys and essentially no other linkage products (**Figure 2**). GABA is directly analogous to N-AcLys, consisting of a primary amino group separated by methylene groups from a carboxyl residue. Reaction of E_2-3,4-Q with GABA and with N-AcLys proceeded at the same rate (change from yellow to red), and yielded products that yielded a single major band by HPLC analysis and exhibited the same absorption spectra with maxima at 510 nm. It is concluded that the product of the reaction between GABA and E_2-3,4-Q is directly analogous to the product of the reaction between N-AcLys and E_2-3,4-Q (**Figure 2**), and is E_2-3,4-Q-1-N-GABA (**Figure 5**).

Estradiol-3,4-Quinone-1-
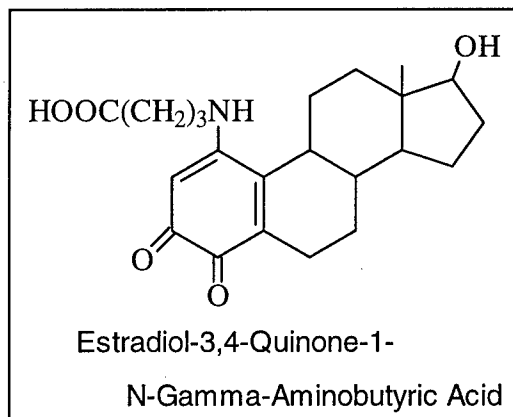
N-Gamma-Aminobutyric Acid

Figure 5

2. Synthesis of E_2-3,4-Q-1-N-GABA is described in detail in Section 'A.4', above.

D. Development of a novel enzyme-linked immunosorbent assay (ELISA) for detection, quantification, and immunochemical characterization of specific monoclonal antibodies

1. Indirect ELISA for anti-E_2-3,4-Q antibodies

The core of the ELISA is a novel coating complex developed as part of the present program (Section B) and consisting of E_2-3,4-Q directly adducted to BSA. The operations are those of a conventional indirect ELISA. The assay was done with a 96-well immunoplate (Maxisorp; Nunc) some wells of which were coated with coating complex by exposure to 100 μL of a solution consisting of 4 micrograms E_2-3,4-Q-BSA per mL carbonate buffer at pH 9.6. Controls wells were coated by exposure to 100 μL of a solution containing 4.5 micrograms BSA per mL carbonate buffer. Coated wells were washed with Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) and then treated with "blocking" buffer, a treatment that prevents nonspecific binding of primary antibody or enzyme-labeled secondary antibody. Blocked wells were washed and then exposed to primary antibody raised against the E_2-3,4-Q epitope linked to protein, for 2 hr at 37 ^{O}C. Subsequently, unbound antibody was washed away and the wells were treated for 1 hr at 37 $^{\circ}$C with horseradish peroxidase (HRP)-labeled secondary antibody specific for the primary antibody. Unreacted antibody was removed by washing with Tris-buffered saline (TBS). The wells were exposed to tetramethylbenzidine substrate for

20 min at room temperature, substrate conversion was stopped by addition of 1M H_2SO_4, and wells were read at 450 nm with an ELISA plate reader.

The indirect ELISA was applied to hybridoma screening, titration of anti-E_2-3,4-Q antibodies, and in modified form, to estimating affinity of anti-E_2-3,4-Q antibodies for the adducted quinone.

2. Competitive ELISA for anti-E_2-3,4-Q antibodies

The competitive ELISA requires two unique components that are unique products of the present program; the coating complex (E_2-3,4-Q-BSA) and the low-molecular-weight competitor (E_2-3,4-Q-GABA). The competitive ELISA is implemented just as the indirect ELISA, with the exception of an additional operation that allows interaction of anti-E_2-3,4-Q antibodies with a range of concentrations of the competitor, under conditions preserving the full functionality of the antibodies. The latter is fulfilled because of the water solubility of E_2-3,4-Q-GABA in contrast to E_2-3,4-Q which has very low solubility in aqueous media. In addition, the competitor provides an excellent mimic of E_2-3,4-Q adducted to cellular and other proteins. Operationally, mixtures of primary antibody and different concentrations of E_2-3,4-Q-GABA are incubated for 2 hr at 37 °C, prior to addition to the coated and blocked wells of the ELISA plate.

Inhibition of antibody binding to coated wells is calculated in reference to coated wells exposed to primary antibody, alone, and is expressed as % inhibition. Inhibition of antibody binding is plotted as a function of log E_2-3,4-Q-GABA concentration. The reciprocal of the concentration of competitor at 50% inhibition is the affinity constant of the antibody.

E. Production and selection of mouse hybridomas secreting antibodies specific for E_2-3,4-Q adducted to proteins

1. Rationale

Mouse antibody-producing cells were induced and harvested according to a protocol that obviates long-term antigen-boosting and repeated sampling and testing of immune sera. A single bolus of immunogen in Freund's complete adjuvant is given subcutaneously and distal to the knee. Two weeks later, raised popliteal lymph nodes containing an array of lymphocyte clones reactive with antigen are easily removed and dissociated to single-cell suspensions for cell fusion. This timesaving approach depends on *ex corporeal* growth and selection of clones secreting high-affinity antibodies.

The first round of hybridoma production was based on immunization with E_2-3,4-Q-OA having a low rate of haptenation (Section 'A.3', above). Antigen-reactive clones were rare and secreted IgM antibodies at low titers. It was concluded that induction of E_2-3,4-Q-specific clones was poor because of the low rate of E_2-3,4-Q substitution (2-3 molecules per molecule of OA) on the carrier protein. In addition, the high solubility of the minimally substituted carrier protein is expected to reduce the amount of immunogen taken up by antigen processing cells of the local lymph nodes.

The second and highly successful round of hybridoma production was based on immunization with cationized OA having a high rate of E_2-3,4-Q substitution (Section 'A.4', above). The mice were given a primary immunization with soluble hapten-carrier complex having five times the haptenation rate as the previous hapten-carrier complex. Subsequently, the animals were given a single booster injection of a highly substituted

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immunogen that remained a fine suspension in aqueous medium. The following section details the work with this protocol.

2. Production and selection of hybridomas secreting E_2-3,4-Q-binding antibodies

Male Balb/c mice were given a single injection of soluble E_2-3,4-Q-GABA-catOA (a novel immunogen developed as part of the present program) in Freund's complete adjuvant. Fifty microliters of inoculum containing 20 micrograms of immunogen were delivered subcutaneously in each hind foot/shank. Three weeks later, the mice were boosted at the same sites with 20 micrograms precipitated immunogen in Freunds incomplete adjuvant. Three days later, popliteal lymph nodes were harvested under sterile conditions and fused with non-secreting P3X63-Ag8.653 cells derived from a myeloma of Balb/c mice. The fusion products were cultured in 96-well plates, in growth medium supplemented with hybridoma cloning factor and a mixture of hypoxanthine, aminopterin, and thymine (HAT supplement). One week later, when clonal growth was observed, HAT supplement was replaced with hypoxanthine/thymine (HT) supplement. Culture medium was harvested when more than 50% of the bottom of a culture well was covered with growing cells. The culture was replenished with fresh medium. Harvested media were analyzed by an indirect ELISA (developed as part of the present program) for the presence of IGG antibody that produced a strong signal (= or > 1.0 absorbance unit) in E_2-3,4-Q-BSA coated wells and only background signal (= or < 0.02 absorbance unit) in BSA coated wells. Ninety-one fusion cultures were screened and thirty-one met the criteria for expansion and further analysis. Six of the thirty-one candidates were exceptional, producing a specific signal = or > 2 and only background signal against BSA, alone. All candidates for further study were grown to high, cell density in 75 cm^2 T flasks and then cryopreserved.

Hybridoma Bd5 one of the six exceptional candidates was selected for cloning. One of these clones was evaluated for antibody production, and its MAb (MAb Bd5) was evaluated for selective binding to E_2-3,4-Q-BSA, and affinity of binding to E_2-3,4-Q-GABA a model of E_2-3,4-Q adducted to proteins. Medium from T-flask cultures of clone Bd5 had a high titer of specific antibody, producing a strong binding signal at a dilution of more than 1000-fold (**Appendix One**). These same antibody preparations displayed absolute selectivity for the coating complex (E_2-3,4-Q-BSA), producing a strong binding signal against coating complex and no signal against BSA, alone (**Appendix One**). The latter is an appropriate control for selective binding since the coating complex was produced by allowing E_2-3,4-Q to adduct spontaneously to BSA in phosphate buffer at pH 6.5.

Antibody binding to the coating complex was studied by competitive ELISA with the water-soluble competitor E_2-3,4-Q-GABA. A plot of log competitor concentration *vs.* % inhibition of antibody binding to the coating complex produced a classical sigmoid curve (**Appendix Two**). The antibody has an affinity constant of 0.5 x 10^8 M^{-1} calculated from the IC50 (2 x 10^{-8} M) determined by competitive ELISA (**Appendix Two**). This calculation was based on the assumption that the concentration of free E_2-3,4-Q-GABA was not significantly altered by antibody binding. According to these criteria, the antibody was determined to be an excellent reagent for immunochemical detection and quantification of the biological marker E_2-3,4-Q adducted to cellular proteins or other macromolecules.

F. Development of novel immunohistochemical protocols for sensitive detection of E_2-3,4-Q as a stable adduct to proteins and other macromolecules of breast epithelial cells collected in a non-invasive manner and fixed on slides, or tissue sections of breast tissue

1. Discussion

Protein coupling reactions (Section 'A.3' and Section 'B.2') and immunochemical assays (Section 'E.2') developed as part of the present program have established that 1) spontaneous formation of stable adducts of E_2-3,4-Q to proteins *in vivo* is feasible, and 2) MAb Bd5 induced against E_2-3,4-Q-catOA meets essential criteria for incorporation into immunohistochemical protocols for detection and quantification of E_2-3,4-Q adducted to proteins or other macromolecules.

E_2-3,4-Q spontaneously forms adducts to OA and BSA (as well as GABA) in phosphate buffer at pH 6.5. Molar ratios of adducted quinone to protein are 2:1 and 5:1, respectively. The basis for selective binding to a limited number of protein sites is unknown. Reaction of the quinone with protein is rapid and produces a distinctly red product that may be seen within 30 minutes after setting up the reaction mixture. The reaction between E_2-3,4-Q and OA or BSA occurs at the same rate as that between the quinone and N-AcLys or GABA, and yields products with the same light absorbance. In view of published studies (4) indicating a high-yield, clean reaction between E_2-3,4-Q and primary amino groups, it is concluded that E_2-3,4-Q readily forms aminoquinone adducts to proteins.

T-flask cultures of hybridoma Bd5 produced high titers of MAb that bound specifically to E_2-3,4-Q-BSA formed by spontaneous reaction of the quinone with BSA, and exhibited no detectable binding to BSA, alone **(Appendix One)**. Competitive ELISA with an aminoquinone competitor produced by spontaneous adduction of E_2-3,4-Q to GABA demonstrated high-affinity binding to the competitor **(Appendix Two)**. Binding of the MAb to the coating complex (E_2-3,4,-Q-BSA) was inhibited 50% by competitor present at 2×10^{-8} M, indicating an affinity constant of 0.5×10^8 M^{-1}, which is effective for immunohistochemical work.

Development of an immunohistochemical protocol for detection and quantification of E_2-3,4-Q adducted to cellular proteins and other macromolecules required optimization of a set of 12-14 variables, some of which must be tested at multiple possible settings. This work was accomplished with E_2-3,4-Q 'positive' sections of rat breast tissue and human MCF-7 cells (breast cancer cells) fixed to slides (analogous to nipple aspirate preparations). E_2-3,4-Q 'positive' tissues and cells were produced by directly exposing tissues and cells to E_2-3,4-Q at 2-10 picomols per mg solution, for 2 hours.

2. Immunohistochemical detection of stable E_2-3,4-Q adducts in paraffin-embedded sections of breast tissue from ACI rats

Nearly all ACI female rats implanted with estradiol, develop breast tumors that are considered an animal model of human breast cancer (5). Work with this material was prompted by 1) the ready availability of paraffin-embedded sections of this material, 2) material demands of experimentation with a diversity of optimization conditions, and 3) the possibility of validating the protocol with a variety of samples from large repositories of paraffin-embedded breast tissue collected from humans. In addition, the results would provide an excellent starting point for work with breast epithelial cells fixed to slides (*e.g.*, nipple aspirate cells).

Protocol optimization studies yielded a great deal of information represented by the following selections. First, epitope recovery in hot citrate buffer destroyed the linkage of E_2-3,4-Q to the primary amino group, and was eliminated from the protocol. Second, endogenous peroxidase activity in the tissue sections was high and required a peroxidase inhibition step in the protocol. Treatment of tissue sections with peroxide/methanol proved to be safe, preserving the integrity of adducted E_2-3,4-Q and blocking endogenous peroxidase activity. Third, the primary antibody could be diluted as little as 1/50 without nonspecific background binding. Fourth, biotinylated secondary antibody produced strong and specific signals at a 1/5000 dilution. Fifth, it was necessary to incorporate a detergent into the wash solution. Sixth, it was possible to combine treatment with primary antibody and blocking of non-specific protein-binding sites in a single overnight treatment at 4°C. Seventh, methanol was the best solvent for delivery of the E_2-3,4-Q-GABA competitor to primary antibody.

The immunohistochemical protocol included the following steps: 1) deparaffinize and hydrate tissue sections, 2) block endogenous peroxidase activity, 3) block endogenous avidin and biotin binding, 4) block nonspecific protein binding, 5) treat with primary antibody, 6) treat with biotin-labeled secondary antibody, 7) treat with horseradish peroxidase (HRP)-streptavidin, 8) treat with tetramethylbenzidine, 9) apply permount, and 10) observe sections, with a microscope.

The first critical test demonstrated intense, antibody binding site-dependent staining of ACI rat breast sections (formalin-fixed, paraffin-embedded) treated briefly with E_2-3,4-Q in phosphate buffer (after deparaffinization and hydration of the sections), and then processed with the immunohistochemical protocol (**Appendix Three**). Both of two paired sections on a single slide were expose to a solution of 5-10 picomols E_2-3,4-Q per mg solution (mimicking concentrations of 4-OHE$_2$ found in human breast tumors). One section was exposed to MAb treated with methanol carrier (**Appendix Three, panels A and C**) and the paired section was treated with MAb exposed to 2×10^{-7} M E_2-3,4-Q-GABA in methanol (**Appendix Three, panels B and D**). It is estimated that only a small fraction of a per cent of the E_2-3,4-Q became adducted to the tissue.

The second critical test demonstrated antibody-dependent, E_2-3,4-Q-specific staining of sections of ACI rat breast (**Appendix Four**). One of two paired sections was exposed to 5-10 picomols E_2-3,4-Q per mg solution (**Appendix Four, panels A and C**) and the second section was exposed to 300-600 picomols of E_2-2,3-Q per mg solution (**Appendix Four, Band D**). Both sections were exposed to untreated MAb Bd5. The slide was then processed with the immunohistochemical protocol. Panels 'A' and 'C' exhibited intense staining with tetramethylbenzidine, and panels 'B' and 'D' exhibited no staining.

3. Immunohistochemical detection of stable E_2-3,4-Q adducts in human MCF-7 cells attached to microscope slides

The protocol described in Section 'F.2' required significant revision for application to whole cells attached to microscope slides. Some of these are described as follows. First, cells were air-dried onto glass slides and fixed with 95% ethanol. Second, air-dried cells required rehydration in buffer and a brief fixation with glutaraldehyde. The latter prevented significant loss of protein caused by required washings with detergent and buffer. Third, the nuclear and cytoplasmic membranes of intact cells fixed to slides pose significant barriers to primary and secondary antibodies as well as to HRP-streptavidin. Consequently, the revised protocol required membrane permeabilization with acetone at

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–15 °C. In addition, peroxidase, biotin, and avidin blocking steps were found unnecessary.

The revised immunohistochemical protocol included the following steps: 1) air-dry and fix cells onto glass slides, 2) hydrate cells and stabilize protein with glutaraldehyde, 3) permeabilize cytoplasmic and nuclear membranes with cold acetone, 4) block nonspecific protein binding, 5) treat with primary antibody, 6) treat with biotin-labeled secondary antibody, 7) treat with HRP-streptavidin, 8) treat with tetramethylbenzidine, 9) apply permount, and 10) observe sections, with a microscope.

A critical test was done with cytospin preparations of normal MCF-7 cells from the same cell suspension **(Appendix Five)**. The cell spots on each of two slides were fixed with glutaraldehyde and then exposed to 1-2 picomols E_2-3,4-Q per mg buffer solution, for 2 hours. The spot on one slide was exposed to MAb treated with methanol carrier **(Appendix Five, panels A and C)** and the paired spot on the second slide was treated with MAb exposed to 10^{-7}M E_2-3,4-Q-GABA in methanol **(Appendix Five, panels B and D)**. Again, it is estimated that only a small fraction of a per cent of the E_2-3,4-Q became adducted to the tissue. The test indicated strong, specific staining of E_2-3,4-Q treated cells.

G. Tissue fixation issues

A recent test indicated that formaldehyde and glutaraldehyde destroy the linkage of E_2-3,4-Q to the primary amino group. This test was done in anticipation of validation studies with benzanthracene- or dioxin-induced MCF-7 cells characterized by markedly increased expression of the estrogen 4-hydroxylasae Cyp1B1. In this case, adducts of E_2-3,4-Q to proteins and other macromolecules are expected to form endogenously, during cell culture. Preliminary tests with acetone fixation of MCF-7 cells previously exposed to exogenous E_2-3,4-Q and then stained with Mab Bd5 demonstrated antibody-dependent staining **(Appendix Six, panel A)**. Control cells exposed to exogenous E_2-3,4-Q and then stained with MAb Bd5 blocked with E_2-3,4-Q-GABA, did not exhibit TMB staining **(Appendix Six, panel B)**. Cellular integrity, however, was compromised as manifested by loss of nuclear delineation and reduced staining intensity. Revised tissue fixation protocols that will stabilize cellular proteins and preserve the integrity of the E_2-3,4-Q epitope must be developed and tested. An effective tissue-fixation protocol is fundamental to studies aimed at validating the application of MAb Bd5 to detection of endogenously formed E_2-3,4-Q adducts to tissue proteins as a biological marker of E-DC.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that estradiol-3,4-quinone (E_2-3,4-Q) spontaneously adducts to primary amino groups of proteins and small molecules, *e.g.*, gamma-aminobutyric acid (GABA) and *N*-acetyl-*L*-lysine, near physiological pH, supporting the possibility of endogenous formation of stable adducts of E_2-3,4-Q to proteins, *in vivo*
- Developed reaction conditions for producing a novel E_2-3,4-Q-specific 1) immunogen, 2) coating complex, and 3) competitive inhibitor
- Developed a novel linker-adduct conjugate by coupling E_2-3,4-Q to GABA, and purified the conjugate by a combination of chloroform extraction, phase separation during rotary evaporation, and solid-phase extraction with a Sep-Pak cartridge

- Developed a novel immunogen for induction of B cell clones specific for E_2-3,4-Q, by over-coupling the linker-adduct conjugate E_2-3,4-Q-GABA to hen egg albumin (OA), in the presence of the carboxyl activator 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy-sulfosuccinimide (S-NHS) which forms a stable ester intermediate with the activated carboxyl group
- Developed a coating complex for enzyme-linked immunosorbent assay (ELISA), by directly reacting E_2-3,4-Q with bovine serum albumin (BSA) thereby producing a physiologically relevant conjugate
- Developed an indirect ELISA and a competitive ELISA for hybridoma screening and immunochemical characterization of candidate MAbs
- Developed a high-affinity MAb (Bd5) specific for E_2-3,4-Q adducted to any protein
- Demonstrated efficient and specific immunohistochemical staining of formalin-fixed or glutaraldehyde-fixed tissues exposed to exogenous E_2-3,4-Q at low picomole quantities
- Determined essential conditions for development of tissue processing required for immunohistochemical detection of stable E_2-3,4-Q adducts formed endogenously in target tissues

REPORTABLE OUTCOMES

A patent disclosure entitled "Stable Adducts of Estradiol-3,4-Quinone to Proteins and Other Macromolecules As A Potential Biomarker of Breast Cancer Risk: Monoclonal Antibody-Based Detection and Quantification in Nipple Aspirate Cells" is under consideration by representatives of the "Intellectual Property Office" of the University of Nebraska Medical Center.

CONCLUSIONS

Work supported by the present "Concept Award" has produced and characterized a novel MAb (Bd5) exhibiting high specificity and affinity for E_2-3,4-Q adducted to any protein. In the indirect ELISA, the antibody produced a strong signal against E_2-3,4-Q-BSA and no signal against BSA, alone. As determined by competitive ELISA, the reaction between the MAb and E_2-3,4-Q-GABA 1) yielded a classical bimolecular plot of E_2-3,4-Q-GABA concentration $vs.$ reduction of ELISA signal, 2) exhibited saturation (100% inhibition of the ELISA signal) at $ca.$ 2×10^{-7} M E_2-3,4-Q-GABA, and 3) had a Ka of 0.5×10^8 M^{-1}. Hybridoma Bd5 secreted the MAb in relatively high abundance as evidenced by the high dilution (1000 to 2000) of T flask culture medium that produced a strong signal against E_2-3,4-Q-BSA in the indirect ELISA. MAb-based immunohistochemical staining produced a strong signal in tissues treated with a few picomoles of E_2-3,4-Q and no signal in tissues treated with a few hundred picomoles of E_2-2,3-Q, indicating effective discrimination of the two estrogen quinones.

MAb Bd5 is a novel probe that offers the opportunity to examine the relationship between non-detoxified E_2-3,4-Q (as evidenced by presence and abundance of stable adducts to proteins) in cells prepared from nipple aspirates or fine-needle aspirates of the breast, and breast cancer risk. This can be achieved through implementation of technologies such as Quantitative Fluorescence Imaging Analysis (QFIA) (6) or Surface-Enhanced Laser Desorption Ionization/tandem mass spectrometry (SELDI/MS/MS) (7) for quantification in individual cells, of E_2-3,4-Q adducts to proteins or E_2-3,4-Q-

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adducted proteins, respectively. QFIA is potentially capable of detecting approximately 100 molecules of adducted protein per cell. MAb Bd5 will make possible acquisition of information needed to determine whether post-translational modification of proteins by non-detoxified E_2-3,4-Q is an important biomarker of breast cancer risk. If the latter is established, the antibody will serve as the keystone of protocols for determining breast cancer risk and efficacy of preventive interventions that may be based on reducing non-detoxified E_2-3,4-Q in breast cells.

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APPENDIX ONE

SPECIFIC BINDING OF MONOCLONAL ANTIBODY Bd5 TO
ESTRADIOL-3,4-QUINONE-BSA, DETERMINED BY INDIRECT
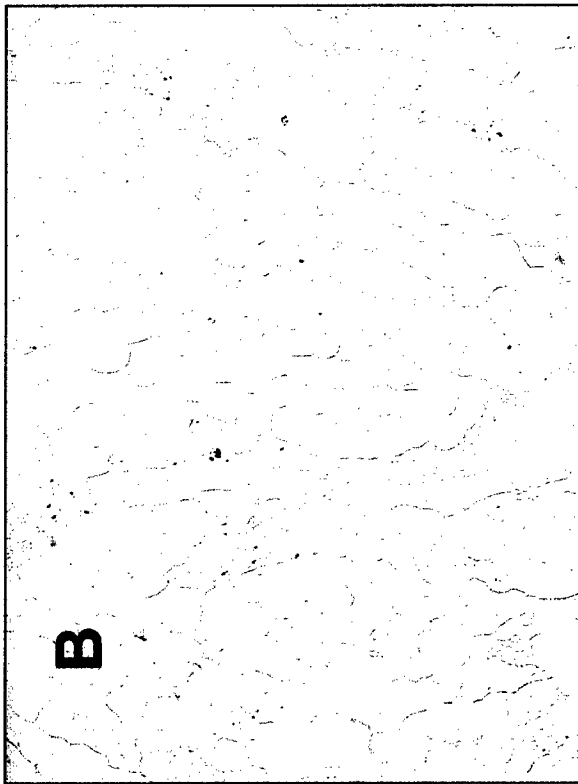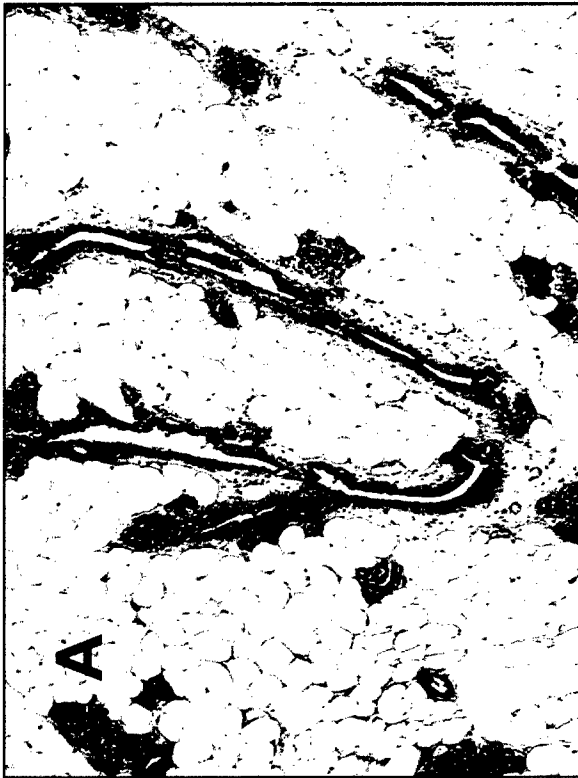ELISA WITH AN HRP-ANTI-MOUSE IGG ANTIBODY
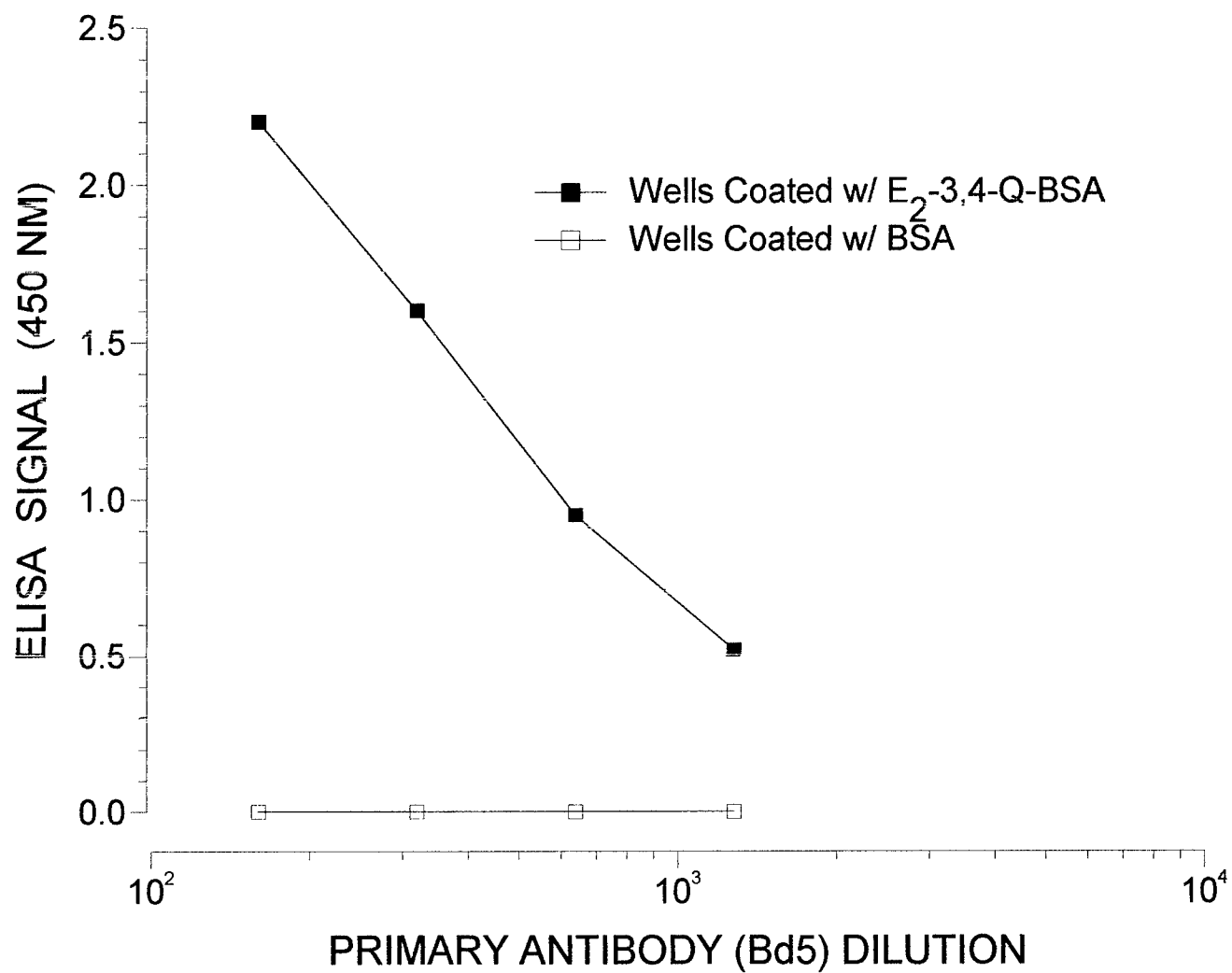
COMPETITIVE ELISA: INHIBITION OF SPECIFIC BINDING OF MAB Bd5 TO E_2-3,4-Q-BSA, BY E_2-3,4-Q-GABA
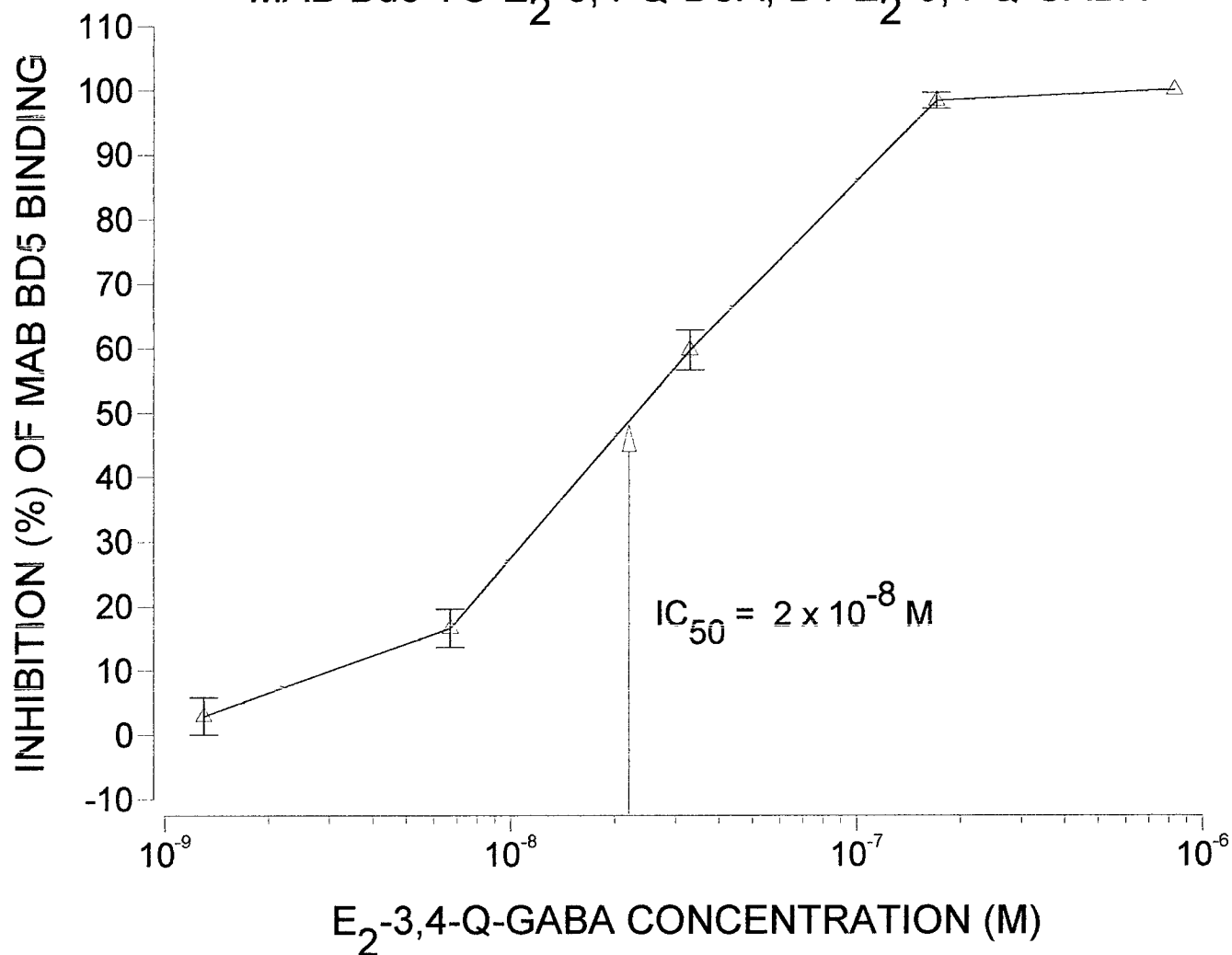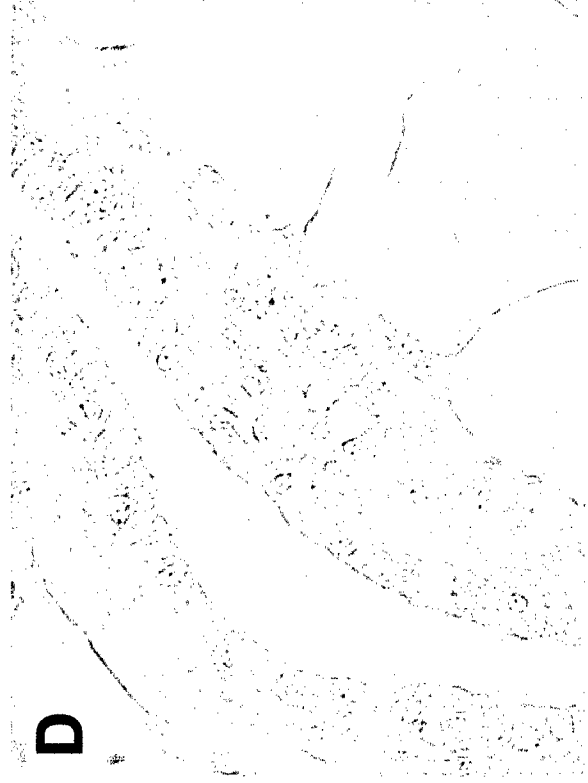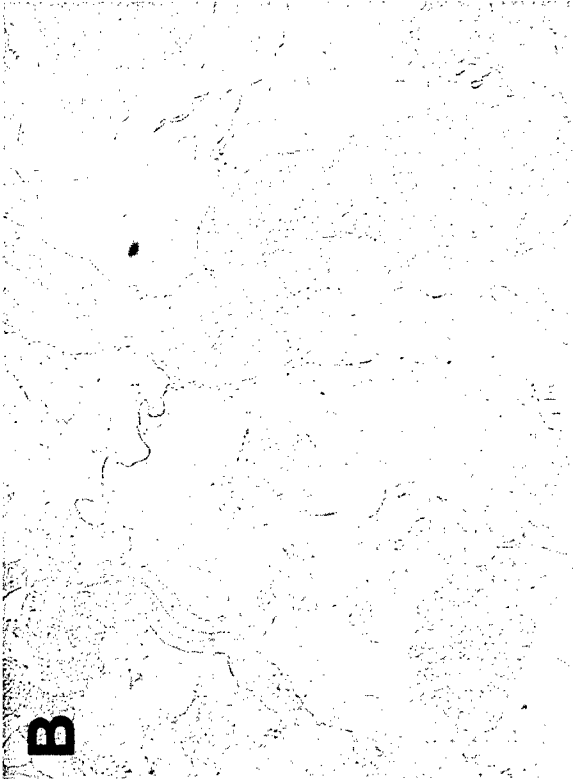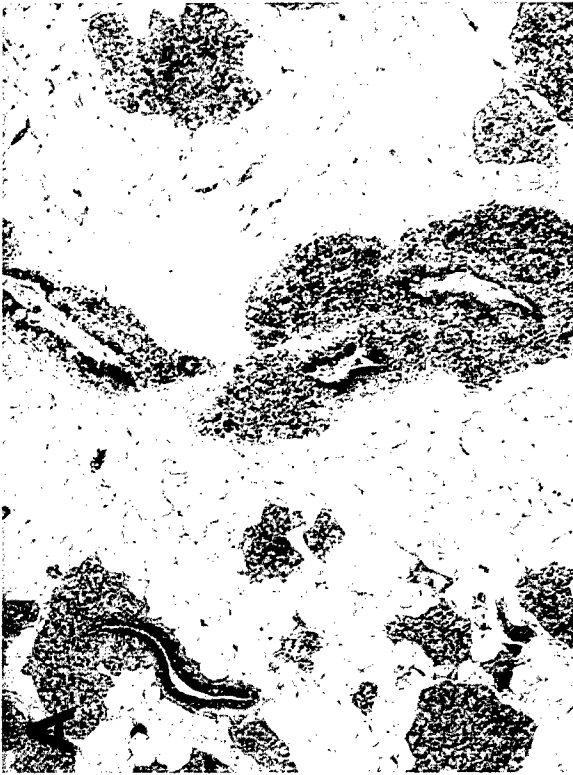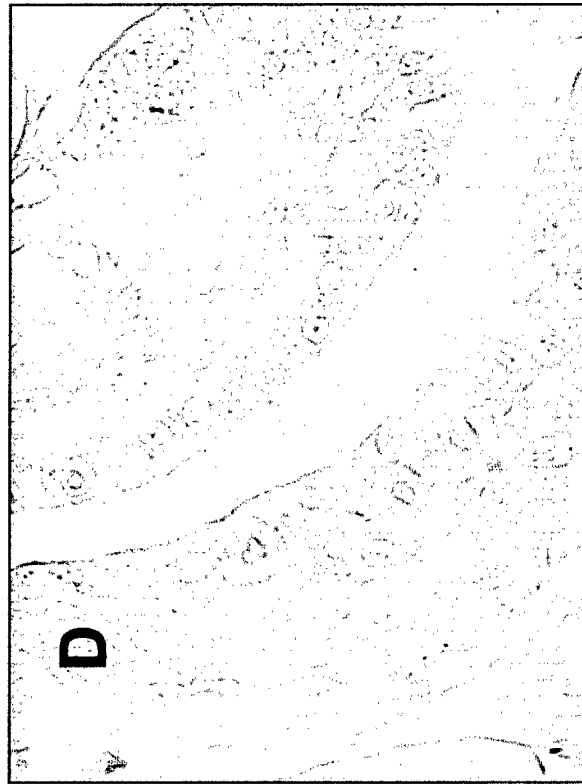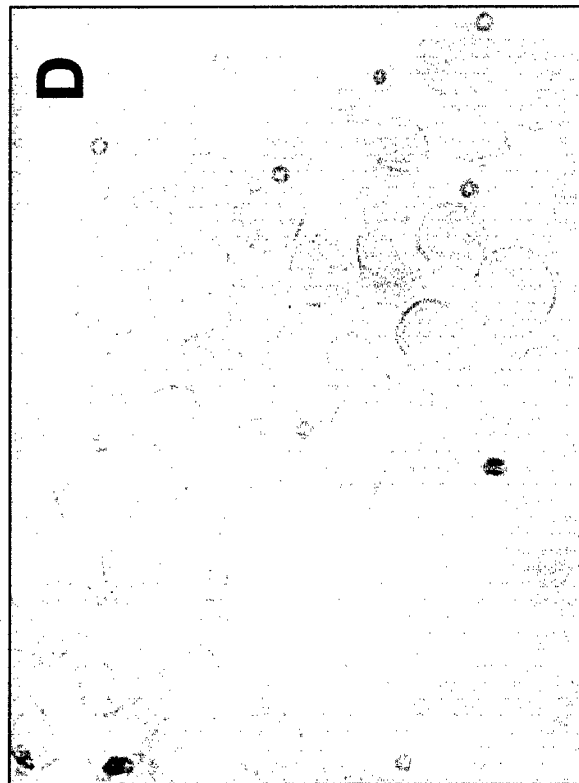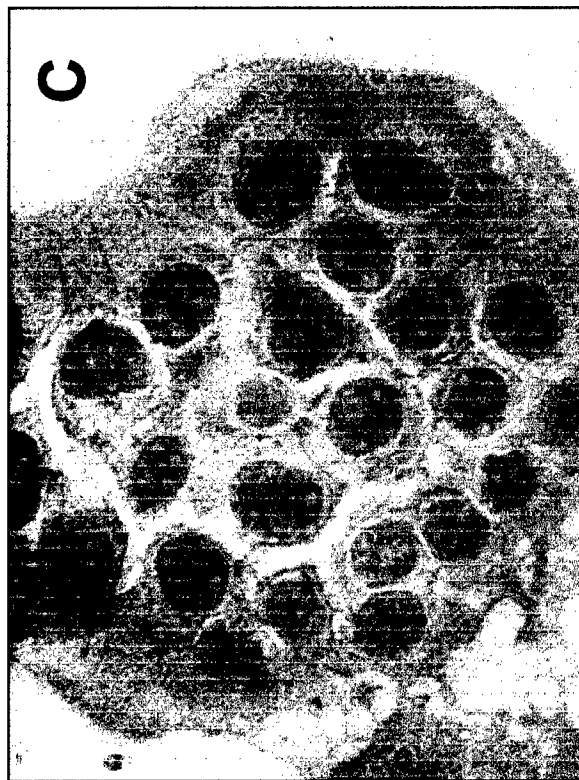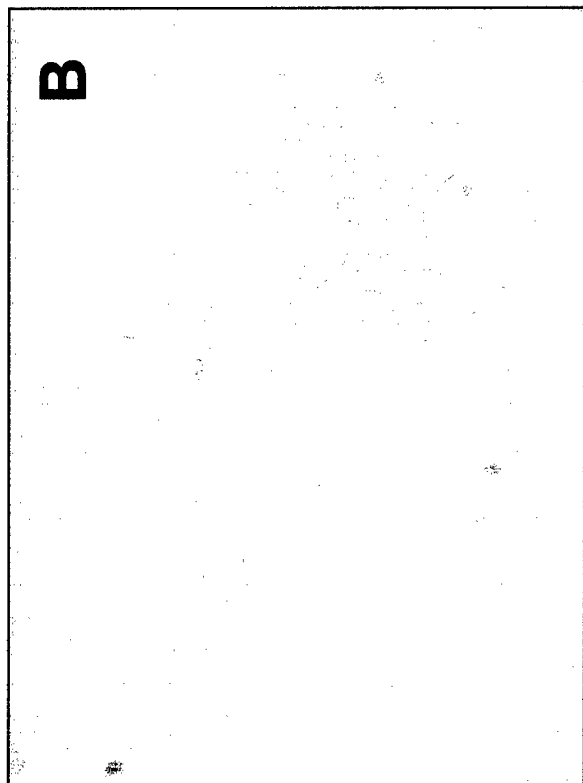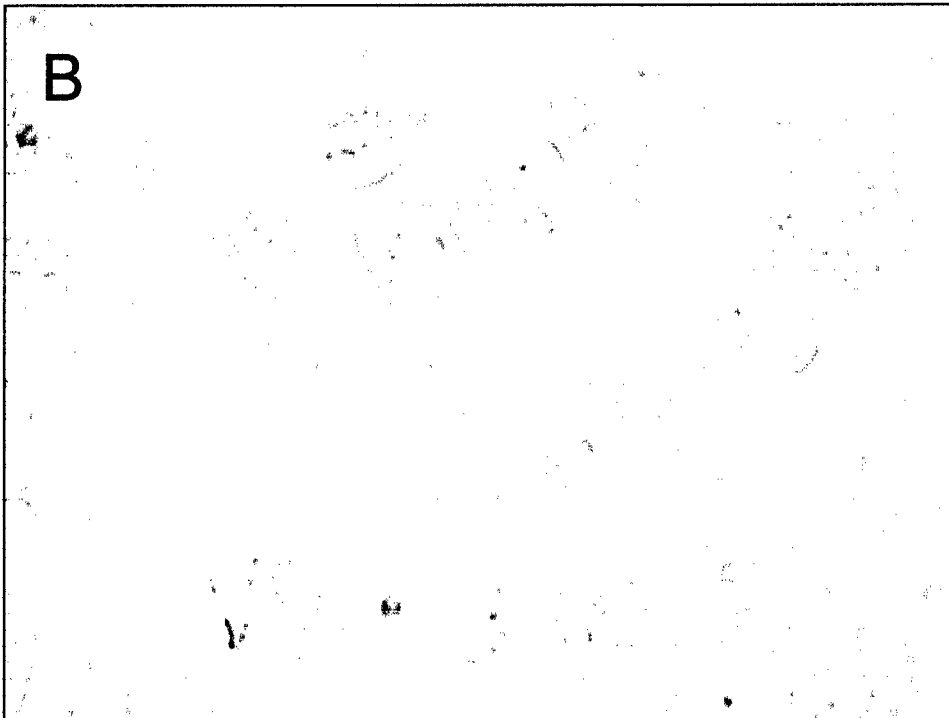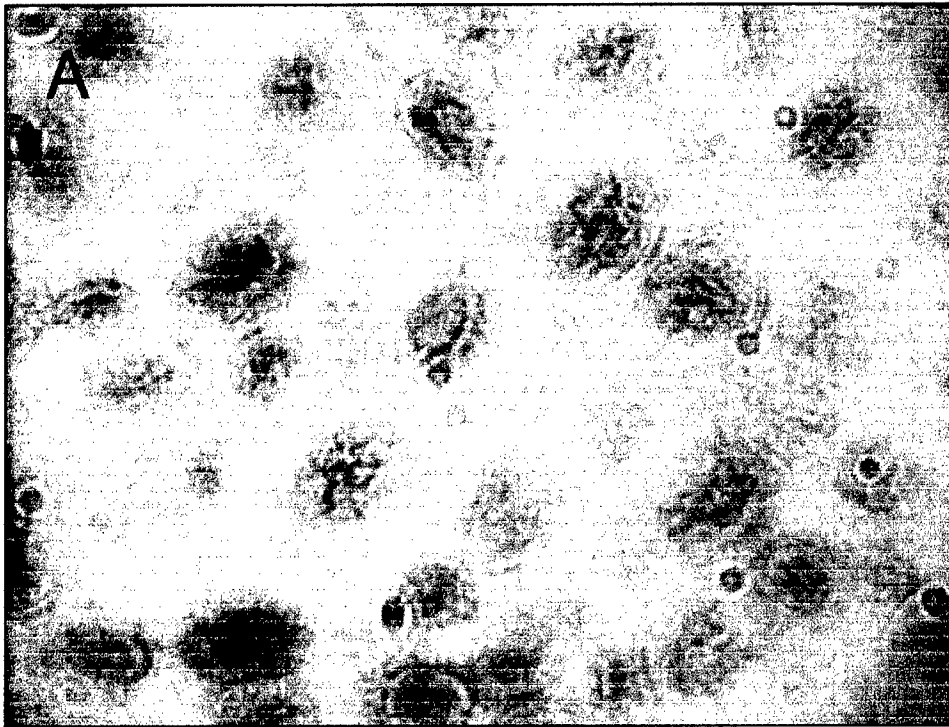
$IC_{50} = 2 \times 10^{-8}$ M

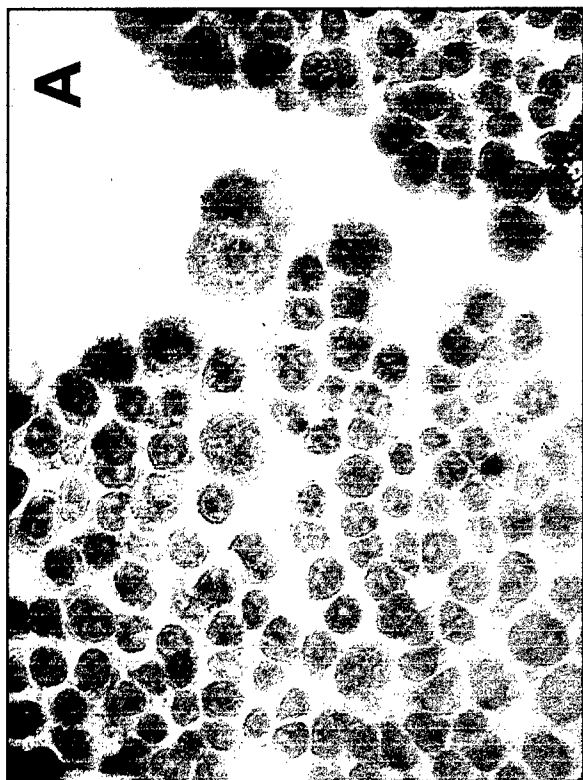INHIBITION (%) OF MAB BD5 BINDING

E_2-3,4-Q-GABA CONCENTRATION (M)

REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   11 Mar 03

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Encl   PHYLIS M. RINEHART
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